Allergology International 66 (2017) S35–S40

Contents lists available at ScienceDirect

Allergology International

journal homepage: http://www.elsevier.com/locate/alit

Original article

Th9 cells induce steroid-resistant bronchial hyperresponsiveness in mice

Mayumi Saeki [a, *], Osamu Kaminuma [b], Tomoe Nishimura [a], Noriko Kitamura [a], Akio Mori [c], Takachika Hiroi [a]

[a] Allergy and Immunology Project, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan
[b] Center for Life Science Research, University of Yamanashi, Yamanashi, Japan
[c] Clinical Research Center for Allergy and Rheumatology, National Hospital Organization, Sagamihara National Hospital, Kanagawa, Japan

ARTICLE INFO

Article history:
Received 2 February 2017
Received in revised form
29 May 2017
Accepted 28 June 2017
Available online 26 July 2017

Keywords:
Airway inflammation
Bronchial asthma
Bronchial hyperresponsiveness
Dexamethasone
Th9 cells

Abbreviations:
Ab, antibody; BALF, bronchoalveolar lavage fluid; BHR, bronchial hyperresponsiveness; Dex, dexamethasone; GR, glucocorticoid receptor; IFN-γ, interferon-gamma; IL, interleukin; MCh, methacholine; OVA, ovalbumin; PBS, phosphate-buffered saline; Rrs, respiratory system resistance; SEM, standard error of mean; TGF-β, transforming growth factor-beta

ABSTRACT

Background: Reduced responsiveness to corticosteroid therapy is a major problem for patients with severe asthma. Although Th9 cells, along with Th2 cells, facilitate antigen-induced airway eosinophilia and bronchial hyperresponsiveness (BHR), the sensitivity of Th9 cell-mediated responses to steroid therapy remains unknown. In this study, we investigated the effect of dexamethasone (Dex) on antigen-induced airway inflammation in Th9 cell-transferred mice.
Methods: Ovalbumin (OVA)-specific Th2 and Th9 cells were polarized from the $CD4^+$ T cells of DO11.10/*RAG-2*$^{-/-}$ mice. BALB/c mice were adoptively transferred with Th2 or Th9 cells and challenged with OVA. Dex treatment was performed twice, at 1 h before and at 24 h after the OVA challenge. Following treatment, the number of inflammatory cells in the bronchoalveolar lavage fluid and the bronchial responsiveness to inhaled methacholine were determined.
Results: In both the Th2 and Th9 cell-transferred mice, substantial accumulation of eosinophils in the lungs and BHR were induced by challenge with the specific antigen. In the Th2 cell-transferred mice, these responses were significantly diminished by Dex treatment. In contrast, neither cellular infiltration nor BHR was affected by Dex treatment in the Th9 cell-transferred mice, although the Th9 cells substantially expressed glucocorticoid receptor α. Accordingly, antigen-induced interleukin-9 expression in the Th9 cells was attenuated by Dex treatment at least *in vitro*. Antigen-induced lung infiltration of infused Th2 cells but not Th9 cells was significantly suppressed by Dex.
Conclusions: In contrast to Th2-mediated responses, Th9-mediated airway inflammation was not affected by Dex. Th9 cells might be involved in the developmental mechanisms of steroid-resistant asthma.

Introduction

Bronchial asthma is a chronic inflammatory disease. Typically, asthma is characterized by reversible airway obstruction, mucus production, and bronchial hyperresponsiveness (BHR) associated with eosinophilic inflammation.[1,2] Th2 cells have been recognized to play a role in the pathogenesis of asthma by secreting cytokines such as interleukin (IL)-4, IL-5, and IL-13.[1–4] These cytokines induce various responses, including immunoglobulin E production, airway eosinophilia, and mucus production.[1–4] Human asthma-like Th2-type airway inflammation could be reconstituted in mouse adoptive transfer models. Thus, antigen-induced eosinophil accumulation in the lungs accompanied by significant BHR has been observed in mice transferred with *in vitro*-differentiated antigen-specific Th2 cells.[5,6]

Th9 cells are a new subset of $CD4^+$ T cells characterized by IL-9-producing activity.[7,8] IL-9 induces mast cell proliferation, goblet cell hyperplasia, BHR, and IL-13 production.[9–12] Therefore, Th9 cells are recognized as potential targets for the treatment of bronchial asthma. Th9 cells can be generated from naive $CD4^+$ T cells *in vitro* by priming with IL-4 and transforming growth factor-beta (TGF-β).[8] In addition, differentiated Th2 cells subsequently produce IL-9 when they are cultured in the presence of TGF-β *in vitro*.[8] Similar to Th2 cell-mediated models, mice transferred with Th9 cells

* Corresponding author. Allergy and Immunology Project, Tokyo Metropolitan Institute of Medical Science, 2-1-6, Kamikitazawa, Setagaya-ku, Tokyo 156-8506, Japan.
E-mail address: saeki-my@igakuken.or.jp (M. Saeki).
Peer review under responsibility of Japanese Society of Allergology.

http://dx.doi.org/10.1016/j.alit.2017.07.001

develop asthma-like airway inflammation characterized by eosinophil infiltration and BHR in response to airway antigen challenge.[13]

In our previous study, we investigated the contribution of eosinophils to BHR using Th2- and Th9-transferred mice in which substantial accumulation of eosinophils in the lungs and BHR were induced by challenge with a specific antigen.[14] An essential and dispensable role of eosinophils in Th2- and Th9-mediated BHR, respectively, was demonstrated by employing eosinophil-deficient mice.[14]

Glucocorticoids have been widely used for asthma therapy owing to their strong attenuating effects on airway eosinophilia by inducing eosinophil apoptosis.[15,16] Regardless of the broad distribution of glucocorticoid receptors (GRs) in the airways, $CD4^+$ T cells are primary targets of glucocorticoids. The number of $CD4^+$ T cells expressing Th2 cytokines in the airways of asthmatic patients was shown to be reduced following glucocorticoid therapy in proportion to the extent of alleviation of their symptoms.[17,18] In animal models, treatment with dexamethasone (Dex) attenuated the Th2 cell-mediated airway inflammation.[19,20]

However, it has been demonstrated that 5–10% of asthmatic patients display severe and glucocorticoid-resistant phenotypes. In this regard, it has not been determined whether Dex treatment is effective for Th9 cell-mediated pathogenesis. Herein, by employing a murine adoptive transfer system for *in vitro*-differentiated Th cells, we investigated the effect of Dex on antigen-specific Th9 cell-induced airway inflammation, including BHR, in comparison to its effect on Th2 cell-mediated pathology.

Methods

Experimental animals

BALB/c mice were purchased from Japan SLC (Shizuoka, Japan). DO11.10/*RAG-2*$^{-/-}$ mice in a BALB/c background were maintained as described previously.[6,14,21] All animal experiments were performed in accordance with guidelines approved by the Animal Use Committee at Tokyo Metropolitan Institute of Medical Science.

In vitro T cell differentiation

Th2 and Th9 cells were prepared as described previously.[6,14,21] In brief, after depletion of erythrocytes, $CD4^+$ T cells were isolated from the splenocytes of DO11.10/*RAG-2*$^{-/-}$ mice using anti-mouse CD4 antibody (Ab)-conjugated magnetic beads and a magnetic cell sorting system (Miltenyi, Bergisch Gladbach, Germany). The cells were cultured in the presence of X-ray-irradiated syngeneic spleen cells as antigen-presenting cells and 0.3 μM ovalbumin (OVA) peptide (OVA323-339 peptide) in Dulbecco's modified Eagle medium-F12/HAM medium (Sigma–Aldrich, MO, USA) supplemented with 10% fetal bovine serum, penicillin, streptomycin, L-glutamine, HEPES, pyruvate, and 2-mercaptoethanol. Th2 differentiation was induced by adding 10 U/mL of recombinant human IL-2 (Shionogi, Osaka, Japan) and mouse IL-4 (PeproTech, NJ, USA), and 10 μg/mL anti-interferon-gamma (IFN-γ) monoclonal Ab (R4-6A2, eBioscience, CA, USA). Th9 cells were differentiated by adding 10 U/mL IL-2 and IL-4, 5 ng/mL recombinant mouse TGF-β (R&D Systems, MN, USA), and 10 μg/mL anti-IFN-γ monoclonal Ab. The cells were cultured for 7 days and then used for the adoptive transfer experiment.

To determine the integrity of polarization, the cells (1×10^5) were incubated with irradiated splenocytes (2×10^5) with or without 0.3 μM synthetic OVA323-339 peptide for 24 h. Then, the total RNA was extracted and subjected to reverse transcription using Super Script III reverse transcriptase (Thermo Fischer Scientific, Waltham, MA, USA) and random primers (Toyobo, Osaka, Japan) for quantitative reverse transcription-polymerase chain reaction for *Il-4*, *Il-5*, *Il-9*, *Il-10*, *Il-13* and *Gra* using TaqMan probes (Thermo Fischer Scientific, Inc.) with a CFX96™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) as described previously.[22] To test the effect of Dex, the compound (10 nM) was added at the start of culture.

Concentrations of cytokines were measured by ELISA (eBiosciences and BioLegend, CA, USA), according to the manufacturer's instructions. Detection limits of IL-4 and IL-9 were 20, and 50 pg/ml, respectively.

Cell transfer and antigen challenge

The BALB/c mice were intravenously injected with cells (1×10^7) suspended in phosphate-buffered saline (PBS). Twenty-four hours after the cell transfer, the mice were challenged with intratracheal injection of OVA solution (25 μL, 15 mg/mL in saline) using a MicroSprayer aerosolizer (Penn Century, PA, USA) under isoflurane anesthesia as described previously.[14] To evaluate the effect of Dex, the mice were subcutaneously injected with either Dex (5 mg/kg) or PBS twice, at 1 h before and at 24 h after the OVA challenge.

Bronchoalveolar lavage fluid (BALF) analysis

Seventy-two hours after the antigen challenge, bronchoalveolar lavage was performed by introducing 3×0.5 mL PBS into the lungs via a tracheal cannula. The number of leukocytes in the BALF was counted using a hemocytometer, and differential cell counts based on morphological criteria were performed for at least 200 cells on a cytocentrifuged preparation after staining with Diff-Quick (Sysmex Corporation, Kobe, Japan). The number of transferred T cells in the BALF was determined by flow cytometry upon staining with anti-CD4-APC-eFluor780 (eBioscience) and anti-KJ1-26-PE (BioLegend).

Measurement of BHR

Seventy-two hours after the antigen challenge, the mice were anesthetized by intraperitoneal injection of 100 mg/kg sodium pentobarbital (Kyoritsu Seiyaku, Tokyo, Japan), and then a 19-gauge cannula was inserted into the trachea. Mechanical ventilation was performed under diaphragmatic perforation using a small animal ventilator (FlexiVent; SCIREQ, Quebec, Canada) at a respiratory rate of 150 breaths/min, a tidal volume of 10 mL/kg body weight, and a positive-end expiratory pressure of 3 cmH_2O. BHR was assessed by measuring the progressive change in respiratory system resistance (Rrs) following inhalation of increasing doses of aerosolized methacholine (MCh; Nacalai tesque, Kyoto, Japan) through an in-line nebulizer.

Statistical analysis

The results are presented as the arithmetic mean ± standard error of mean (SEM). Statistical analysis was performed using Student's *t*-test or one-way analysis of variance and Dunnett's multiple comparison test. *P*-values less than 0.05 were considered to indicate statistical significance.

Results

Cytokine expression of differentiated T cells in vitro

To evaluate the phenotypes of *in vitro*-differentiated Th2 and Th9 cells, their cytokine mRNA expression profiles, and concentration of cytokines in the culture supernatant were examined. Th2